EXPRESSION OF RECOMBINANT HUMAN ACETYLCHOLINESTERASE IN TRANSGENIC PLANTS

REFERENCE TO RELATED APPLICATIONS

This application claims an invention that was disclosed in Provisional Application Number 60/190,440, filed March 17, 2000, entitled "EXPRESSION OF RECOMBINANT HUMAN ACETYLCHOLINESTERASE IN TRANSGENIC TOMATOES." The benefit under 35 U.S.C. § 119(e) of the United States Provisional Application is hereby claimed, and the aforementioned application is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The invention pertains to the field of transgenic plants. More particularly, the invention pertains to the expression of a recombinant form of human acetylcholinesterase in transgenic plants.

DESCRIPTION OF RELATED ART

Acetylcholine (ACh) is one of the major signaling molecules in metazoans, functioning mostly as a neurotransmitter in chemical synapses between neurons and in neuromuscular junctions. To ensure a discrete "all-or-none" response across the synapse, the release of ACh is tightly controlled and the neurotransmitter is efficiently removed by the hydrolyzing enzyme, acetylcholinesterase (AChE). In humans, AChE is encoded by a single gene which yields, through alternative splicing of its pre-mRNA, three polypeptide isoforms having distinct C-termini. See Soreq *et al.*, *Proc. Nat. Acad. Sci. U.S.A.* 87: 9688-9692 (1990); Ben Aziz-Aloya *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90: 2471-5 (1993); GenBank Accession No. M55040; and U.S. Patent No. 5,595,903. The complete disclosure of each of the foregoing references is hereby incorporated herein by reference.

Various compounds are well known to inhibit the hydrolyzing activity of AChE. Exposure to such anti-AChE agents leads to over-stimulation of cholinergic pathways,

causing muscular tetany, autonomous dysfunction and potentially death. While some naturally occurring AChE inhibitors are very potent, human exposure to them is rare. However, man-made anti-AChE compounds, especially organophosphates (OPs), are widely used as pesticides and pose a substantial occupational and environmental risk. Even more ominous is the fear of deliberate use of OPs as chemical warfare agents against individuals or populations.

Current medical interventions, in the case of acute exposure to anticholinesterase agents, include use of the muscarinic receptor antagonist, atropine, and oximes to reactivate the OP-modified AChE. The reversible carbamate, pyridostigmine bromide, is also used as a prophylactic. However, these conventional treatments have limited effectiveness and have serious short and long-term side effects. In fact, the routine treatments, while successfully decreasing anticholinesterase-induced lethality, rarely alleviate post-exposure delayed toxicity, which may result in significant performance deficits, and even permanent brain damage.

A different approach in treatment and prevention of anti-AChE toxicity seeks to mimic one of the physiological lines of defense against such agents present in mammals. Butyrylcholinesterase (BuChE) is a serum cholinesterase with a broad hydrolytic spectrum that provides protection against a variety of AChE inhibitors. A similar end may be achieved by a variant of AChE found on the membranes of erythrocytes. Both enzymes are believed to serve as circulating scavengers for anti-AChE agents in protection of the vital synaptic AChE. Therefore, administration of cholinesterases could boost their natural potential to counter-act the toxic effects of anti-cholinergic agents. The efficacy of this treatment to protect against a challenge of OPs was tested in a variety of animal models such as mice, rats, guinea pigs, and primates, and was found to be comparable to or better than the currently-used drug regimens in preventing OP-induced mortality without any detrimental side-effects.

Enzyme therapy has the additional benefit of the relatively long half-life time (several days) of the injected enzymes in the blood stream, making it especially useful for prophylaxis. In the foregoing experiments, cholinesterases purified from human or animal blood were used. To be effective, the stoichiometry of cholinesterase to inhibitor must be

close to unity. Hence, large amounts of pure, properly folded, stable enzymatic preparations that are free of mammalian pathogens are needed, if enzyme therapy is to be feasible.

Genetically engineered plants have recently been recognized as one of the most cost-effective means for the production of useful recombinant proteins and pharmaceuticals. Therefore, we examined the use of transgenic plants as a cost-effective and safe alternative to the production of human acetylcholinesterase (hAChE) from blood or cell cultures, herein providing the first demonstration of the expression in plants of a key protein component of the nervous system of humans.

SUMMARY OF THE INVENTION

Briefly stated, the invention includes one or more plant cells comprising a polynucleotide that encodes a human acetylcholinesterase.

An embodiment of the invention includes a method of making a transgenic plant that is capable of expressing a physiologically active human acetylcholinesterase, comprising the steps of introducing into at least one plant cell a polynucleotide that encodes a human acetylcholinesterase, and regenerating from the plant cell a transgenic plant that is capable of expressing a physiologically active human acetylcholinesterase in at least one tissue type of the transgenic plant.

Another embodiment of the invention includes a method of making a physiologically active human acetylcholinesterase, comprising the steps of introducing into at least one plant cell a polynucleotide that encodes a human acetylcholinesterase, regenerating from the plant cell a transgenic plant that is capable of expressing a physiologically active human acetylcholinesterase in at least one tissue type of the transgenic plant, and isolating or purifying from the transgenic plant or a part thereof a physiologically active human acetylcholinesterase.

Another embodiment of the invention includes a method of treating a victim of acetylcholinesterase poisoning, comprising the step of administering a therapeutic amount of a physiologically active human acetylcholinesterase expressed in plant tissue.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 shows a graphic map of pTM036, the pGPTVkan derivative construct used in the generation of transgenic tomato plants that constitutively express hAChE-E4.

Fig. 2 shows a bar graph depicting high activity of hAChE in transgenic tomato lines.

Fig. 3 shows substrate inhibition of recombinant hAChE obtained from transgenic plants.

Fig. 4 shows an inhibition profile of AChE obtained from transgenic plants (diamonds), human erythrocytes (circles) and transgenic mice (squares).

Fig. 5A shows a graph of data indicating that a recombinant hAChE derived from transgenic plants is labile at relatively high temperatures.

Fig. 5B shows a graph of data indicating that a plant-derived hAChE is relatively stable at room temperature.

DETAILED DESCRIPTION OF THE INVENTION

DNA constructs and plant transformation

A cDNA encoding human AChE exons II, III and IV was amplified from the plasmid pAChE-E4 (see Sternfeld *et al.*, *J. Neurosci.* 18: 1240-1249 (1990), the complete disclosure of which is hereby incorporated herein by reference) via the polymerase chain reaction (PCR), according to standard methods, which are well known in the art, using the following primers:

AChE-*Nco* - (5'-GATATCTGCAGCCATGgctAGGCCCCCGC) (SEQ ID NO:1)

AChE-*Kpn* - (5'-CggtaccTATCAGGTaGCGCTGAGCAATTTG) (SEQ ID NO:2)

The lower case letters in the foregoing primer sequences represent bases that were introduced to create restriction sites for cloning the gene into plant expression vectors. The PCR product was cloned and sequenced using well known methods. An *Nco I-Kpn I* fragment from a partial digest of pAChE-E4 was cloned into pIBT210.1 (see Haq *et al.*, *Science* 268: 714-716 (1995), the complete disclosure of which is hereby incorporated

herein by reference) behind a CaMV 35S promoter and the 5' UTR of Tobacco Etch Virus, and in front of the 3' UTR of the soy bean *vspB* gene to form pTM034 (Figure 1, SEQ ID NO:3), according to standard methods, which are well known in the art. A *Hind III-Eco RI* fragment containing the plant expression cassette was then cloned into the T_i plasmid derivative pGPTV-Kan to form pTM036 (SEQ ID NO:4), using standard methods that are well known in the art. This plasmid was then transferred to *Agrobacterium tumefaciens* strain EHA105, and was used in the subsequent transformation of the *Lycopersicum esculentum* cultivar referred to as "Micro-Tom," as described by Meissner *et al.* in *Plant J.* 12: 1465-1472 (1997), the complete disclosure of which is hereby incorporated herein by reference.

Genomic PCR, DNA and RNA blot analysis

Screening by genomic PCR was performed on 0.8 μg total DNA isolated from kanamycin resistant plants, using the AChE-*Nco* and AChE-*Kpn* primers, according to well known methods. For DNA blot analysis, total DNA was prepared, digested with *Nco I*, and the digested DNA (~20 μg) was resolved by electrophoresis, transferred to a nylon hybridization membrane, and hybridized to a digoxigenin-labeled probe, according to standard methods, which are well known in the art. The digoxigenin-labeled probe was synthesized using the following primers:

AChE585for (5'-CGAGAGGACTGTGCTGGTGTC)

AChE1374rev (5'-GTCGCCCACCACATCGCTC)

Hybridization and detection were performed according to well known methods. Total RNA was isolated and 5 μg samples were resolved by denaturing formaldehyde gel electrophoresis and transferred to nylon hybridization membranes, according to well known methods.

Acetylcholinesterase assays and protein determination

Plant samples were homogenized in the presence ice-cold extraction buffer (100 mM NaCl, 25 mM Tris, 0.1 mM EDTA, 10 μg/ml leupeptin, pH 7.4, 3 ml per 1 g tissue) using ceramic beads in a bead-beater, and cleared supernatants were collected followed by

centrifugation (14,000 rpm). Scaled-down microtiter plate Ellman assays were performed, according to standard methods, which are well known in the art. Cleared extracts (~20 μl) were incubated for 30 minutes at room temperature with 80 μl assay buffer (0.1 M phosphate buffer, pH 7.4) with or without $2x10^{-5}$ M 1,5-bis(allyldimethylammiumphenyl)pentan-3one dibromide (BW284c51), which is a specific inhibitor of mammalian AChE. At the end of the 30 minute incubation period, 100 μl of 1 mM 5-5'-dithio-bis(2-nitrobenzoate) (Ellman's reagent) and 2 mM acetylthiocholine in assay buffer was added. Hydrolysis was monitored by measuring optical density at 405 nm at 5 minute intervals for 30 minutes, using a microtiter plate spectrophotometer, plotted against time, and initial rates were calculated from the slope of the linear portion of the graph. Net hydrolysis rates were calculated by subtracting the rates measured in the presence of BW284c51 from those obtained in its absence. To determine the K_m, the concentration of the acetylthiocholine substrate in the Ellman's reagent was varied in the range of 0.05-50 mM.

Inhibition curves were obtained by performing the Ellman assay with 1 mM acetylthiocholine in the presence of the indicated concentrations of diethyl p-nitrophenyl phosphate (paraoxon), neostigmine, phehylmethylsulfonyl fluoride (PMSF) or tetraisopropyl pyrophosphoramide (Iso-OMPA). To determine K_I of BW284c51, assays were performed in the presence of 1, 0.33 and 0.25 mM acetylthiocholine, and the inhibitor at 10^{-4} to 10^{-10} M. Results were then analyzed according to the method of Ordentlich *et al.* (see Ordentlich *et al.*, *J. Biol. Chem.* 268: 17083-17095 (1993), the complete disclosure of which is hereby incorporated herein by reference). In these experiments, acetylcholinesterase from human erythrocytes was used.

To evaluate the heat stability of the enzyme, plant extracts were incubated for 30 minutes at the indicated temperatures and then assayed as described above. Stability of the enzymatic activity was determined at 4 degrees C and at 25 degrees C by incubating plant extracts at the respective temperatures and assaying samples at the indicated time points.

A cDNA encoding exons 2-4 of the human AChE29 gene was inserted into a plant expression cassette driven by the constitutive cauliflower mosaic virus 35S promoter.

Referring now to Figure 1, a graphic map is shown of pTM036, the pGPTVkan derivative construct used in the generation of transgenic tomato plants that constitutively express hAChE-E4. Empty arrowheads denote positions of the PCR primers AChE-*Nco* and AChE-*Kpn* used for amplification of the full length coding region of hAChE-E4. Filled arrowheads denote the positions of the PCR primers AChE585for and AChE1374rev used for the generation of DIG-labeled probe.

We used *Agrobacterium tumefaciens* to construct the tomato explants, and regenerated 27 kanamycin resistant tomato lines. We screened the transformants for the insertion of the recombinant human gene AChE-E4 by PCR. Twelve out of 17 plants tested were positive for the appropriate gene insertion event. The product of the AChE-E4 construct was previously demonstrated to be a monomeric soluble protein, which is fully active in acetylcholine hydrolysis. Therefore, we screened the putative transgenic plants for the expression of specific acetylcholinesterase activity in the soluble protein fraction of leaf extracts of kanamycin-resistant lines.

Referring now to Figure 2, kanamycin-resistant lines were assayed for specific esterase activity (*i.e.*, total minus activity in the presence of the inhibitor BW284c51) in leaves by the method of Ellman, using acetylthiocholine as a substrate. Protein samples from the indicated transgenic plant lines (AChE-53, 54, 62, 68 and 83), untransformed plant (UT) and a commercially available preparation of AChE from human erythrocytes (E5) were resolved on a non-denaturing gel which was then stained for AChE activity. Plant-derived AChE migrates as a discrete band in non-denaturing gel electrophoresis. On a per soluble protein basis, high activity, comparable to a third of the activity present in mammalian brain and five times more than that present in muscles, was registered in several of the lines, including AChE-53, AChE-54, AChE-62 and AChE-68. In these lines, activity was on the order of 100 mU/g leaf tissue (fresh weight). Acetylcholinesterase present in the transgenic lines appeared as a discrete band in non-denaturing polyacrylamide gels stained for cholinesterase activity. This result demonstrates the apparent uniformity of the protein produced by the plants. No activity was detected in the untransformed line, or in line AChE-83. Unexpectedly, in contrast to the sharp bands of the plant derived recombinant enzyme, the activity of the commercially available preparation of AChE from human erythrocytes appeared as a diffuse smear.

DNA blot analysis revealed that three of the lines that express high levels of activity, AChE-54, AChE-62 and AChE-68, each have one copy of the hAChE-E4 gene inserted in their genomes. Total DNA was isolated from the indicated lines, digested with *Nco I*, resolved by agarose gel electrophoresis, blotted to nylon membrane and probed with digoxigenin-labeled probe, according to well known methods. Referring to Figure 2, AChE-83, a transgenic line that does not exhibit AChE activity, has at least two copies of the gene inserted in its genome. However, in this line, the mRNA encoding hAChE-E4 failed to accumulate to detectable levels, as demonstrated by RNA blot analysis, suggesting that transgene silencing in this line might have occurred. RNA blot analysis of several kanamycin-resistant tomato lines indicated that mRNA accumulated to similar levels in all the other lines that were tested. Total RNA was isolated from the indicated lines, resolved by agarose gel electrophoresis, blotted to nylon membrane and stained with methylene blue. The membrane was then probed with AChE specific DIG-labeled probe.

Kinetic properties of the plant-produced recombinant enzyme

We calculated the K_m of the plant-derived enzyme for four of our expressing lines to be 0.44±0.10 mM (Figure 3, inset). This value is similar to that reported for the same molecular form of the enzyme expressed in injected oocytes of *Xenopus laevis* and also to those reported for other forms of the human enzyme. Hydrolysis was inhibited by the presence of substrate at high concentration (Figure 3), as previously reported for native and recombinant AChE. Enzyme activity was assayed in the presence of acetylthiocholine at 0.05-50 mM, and hydrolysis in the presence of the inhibitor BW284c51 was subtracted at each concentration. A representative high expression line (AChE-54) is shown in Figure 3. The inset of Figure 3 shows Lineweaver-Burk analysis for the determination of the K_m for four lines: AChE-53 (squares), AChE-54 (diamonds), AChE-62 (triangles) and AChE-68 (crosses).

AChE inhibitors of various classes, including the reversible inhibitors neostigmine (a carbamate), BW284C51 (an AChE-specific bisquaternary inhibitor), as well as the irreversible inhibitors paraoxon (an organophosphate, the activated form of the pesticide parathion) and PMSF (a general serine hydrolase inhibitor) can inhibit the plant derived recombinant AChE (rAChE), and the inhibition profile is very similar to that of a

commercially available preparation of human AChE derived from erythrocytes (Figure 4). The K_I calculated for BW284c51 is 16 nM, which is in close agreement with the values for the recombinant human synaptic enzyme transiently expressed in mammalian cell cultures (10 nM) and for the erythrocyte form (5 nM). As expected, the butyrylcholinesterase-specific organophosphate, Iso-OMPA, had no effect on either the plant-derived or the erythrocyte-derived enzyme preparations (up to 100 μM), and only partial inhibition was registered at 10 mM (Figure 4). The plant-derived E4 enzyme was somewhat less susceptible to paraoxon than an equivalent recombinant enzyme obtained from transgenic mice (Figure 4).

The plant-derived hAChE in total soluble protein extracts retained 50% of its initial activity after incubation at 42 degrees for at least 30 minutes (Figure 5A). Crude leaf extracts were incubated at the indicated temperatures for 30 minutes and then subjected to Ellman's AChE assay. Incubation of plant extracts at room temperature (~25 degrees C) resulted in gradual loss of AChE activity, with 20% residual activity remaining after 25 hours (Figure 5B). The activity was very stable at 4 degrees C, with only 20% loss after 24 hours (Figure 5B). Crude leaf extracts were incubated at 4 degrees C or at 25 degrees C for the indicated time periods and then assayed for AChE activity.

Types of cholinesterases that can be expressed in plants

Traditionally, cholinesterases are classified as either acetylcholinesterase (EC 3.1.1.7, AChE) or as butyrylcholine hydrolases (EC 3.1.1.8, BChE, formerly referred to as pseudo-acetylcholinesterase) on the basis of their substrate specificity. While BChE can efficiently hydrolyze substrates with a longer acyl group, the catalytic efficiency of AChE is limited to acetylcholine and, to a lesser degree, propionylcholine. More recently inhibitors have been identified that can selectively inhibit the two types of cholinesterases.

The genes encoding AChE and BChE from several mammals, including humans, have been cloned. Cholinesterases from non-vertebrates and lower vertebrates, even when possessing several different genes, have mixed characteristics. A further complication of the molecular picture is presented by the alternative splicing that the transcript of the AChE gene can undergo leading, in mammals, to three distinct isoforms. These isoforms

share a common N-terminal catalytic domain, but diverge in their C-termini, which impact their quaternary structure and membrane association.

The catalytic distinction between the enzymes is not restricted to acyl-choline substrates but to other types of esters. Thus, BChE can catalyze the hydrolysis of cocaine whereas AChE cannot. On the other hand, it was recently demonstrated that the erythrocyte form of AChE can hydrolyze heroin (3,6-diacylmorphine) to morphine, while BChE can hydrolyze heroin only to the intermediate 6-NAM (6-monoacetylmorphine). Interestingly, the synaptic isoform of AChE cannot hydrolyze heroin, making heroin hydrolysis the first reported catalytic distinction between the different isoforms of AChE.

The literature on the non-cholinergic functions of cholinesterases, and especially of AChE, is becoming richer all the time. These proteins apparently play important roles in the developing nervous system and its maintenance, especially in directing the growth of neurons and establishing synaptic connections. The different isoforms have distinct roles through their different C-termini. For example, addition of the synaptic isoform of AChE to cultured neurons has a marked activation effect on neurite outgrowth, and a similar effect has been noted in transgenic frog embryos. In contrast, frog embryos expressing soluble forms of the enzyme do not exhibit such effects.

These small nuances make all of these different isoforms valuable, and we anticipate that plant production of them will be useful for many different ends, including, but not limited to, the following: 1) scavengers of anticholinesterase agents including organophosphates; 2) the hydrolysis of cocaine and heroin in treatment of cases of overdose intoxication by drug abusers; and 3) regeneration of damaged neuronal tissue.

Optimization of the coding sequence of hAChE-E4 for expression in plants

In most cases, the accumulation of foreign proteins in transgenic proteins is a desirable objective, as it tends to maximize yield and reduce costs of production. Accumulation of proteins is a complex function of many factors that effect synthesis and degradation. By "synthesis" we mean all the biochemical steps which lead to the formation of a mature protein, from transcription of a gene, accumulation of mRNA, translation of messages, localization of products, and many co- and post-translational

modifications. Not all of these steps can easily be controlled directly (some, for example, are inherent to the polypeptide in question) and, as yet, not all can be manipulated to enhance accumulation. However, experience has shown that certain optimization measures can have a profound effect on the overall levels of foreign protein accumulation in plants.

Up to the translation stage, the expression of a gene is dependent on the nucleotide sequence not only of the control elements, such as promoter, enhancer elements and 3' sequences, but also on the coding region as well. Molecular cues are encoded by the nucleic acid sequence to allow molecular events, such as termination of transcription, splicing of intervening sequences, rapid turnover of mRNA, and its translatability. Many of these features are common to many different types of organisms, while others are specific for plants, including, for example, intron splice sites, plant-specific RNA stabilizing sequences, and even plant specific biases in codon usage. Thus, optimization of gene sequences entails conforming the coding sequence to those of plant genes.

Numerous methods for the optimization of DNA sequences for increased expression in plants are well known in the art. For example, see U.S. Patents No. 6,180,774; 6,166,302; 6,121,014; 6,110,668; 6,075,185; 6,051,760; 6,043,415; 6,015,891; 6,013,523; 5,994,526; 5,952,547; 5,880,275; 5,877,306; 5,866,421; 5,859,347; 5,859,336; 5,689,052; 5,633,446; 5,625,136; 5,567,862; 5,567,600; 5,545,817; 5,500,365; and 5,380,831, the disclosures of each of which are hereby incorporated herein by reference.

Analysis of the cDNA of hAChE-E4 to assess its suitability for expression in plants

Although we present herein an example wherein the hAChE gene is expressed in tomato plants, the tomato serves here only as a model organism. The expression of hAChE in all major crop plants is intended to be within the scope of the present invention, including (but not restricted to): dicotyledonous plants, such as, for example, tomato, potato, tobacco, legumes (*i.e.*, soybean, peanut, alfalfa), and sweet potato. These plants are typically engineered by *Agrobacterium* transformation, various suitable methods for which are well known in the art. Monocotyledonous plants are also intended to be within the scope of the present invention, including (but not restricted to): maize, rice, wheat,

and barley. These plants are typically engineered by biollistic transformation, various suitable methods for which are well known in the art.

The hAChE-E4 nucleotide sequence includes a total of 574 codons and has an A+T content of 34.8%. Codon use in hAChE-E4 generally is unfavorable for expression in dicots, but acceptable for expression in monocots. In summary, 3.6% of the codons are monocot-unfavorable (including Arg - 17.5%, Lys - 42.9% and Ser - 15.6%), while 12.7% are dicot-unfavorable (including Arg - 72.5%, Gly - 32.8, Pro - 17.6% and Thr - 32%), when favorability is defined as making up less than 10% of codon choice for a particular amino acid. Monocot and Dicot preferences were analyzed separately, so as to reveal any potential monocot vs. dicot problems. Tables I and II below summarize total codon use:

Table I - Dicot

AA	DNA	Unfavorable	Total	%
Ala	GCG	5	53	9.5
Arg	CGA	7	40	72.5
	CGC	8		
	CGG	14		
Gly	GGG	19	58	32.8
Leu	CTA	1	67	1.5
Pro	CGG	9	51	17.6
Ser	TCG	2	32	6.3
Thr	ACG	8	25	32
Other				
Total		73	574	12.7

Table II - Monocot

AA	DNA	Unfavorable	Total	%
Arg	CGA	7	40	17.5
Ile	ATA	0	9	0
Leu	CTA	1	67	1.5
	TTA	0		
Lys	AAA	3	7	42.9
Ser	AGT	5	32	15.6
Val	GTA	5	53	9.4
STOP	TAA	0	0	0
Other				
Total		21	574	3.6

Based on the data in the foregoing tables, it is evident that some optimization of the native hAChE-E4 nucleotide sequence is desirable, particularly if the gene is to be expressed in dicots. Thus, we present herein an example of a synthetic DNA sequence encoding human acetylcholinesterase that is optimized for expression in plants, referred to herein as SEQ ID NO:5.

Purification of plant-produced cholinesterases

While for some of the potential applications of cholinesterases, no purification would be necessary (*e.g., in-vivo* bioremediation), and for other applications only partially purified preparations of the enzymes would be necessary (*e.g.*, certain industrial uses, oral administration, topical applications in creams, *etc.*), for other applications relatively pure enzymes are preferable, and may be required. This is especially true for treating individuals by intra-venous or intra-muscular injections of cholinesterases.

Several published procedures for the purification of acetylcholinesterase are known in the art. See, for example, Fischer *et al., Biotechnol. Appl. Biochem.* 21: 295-311 (1995) and Heim *et al., Biochim. Biophys. Acta* 1396: 306-319 (1998), the complete disclosures of which are hereby incorporated herein by reference. A large scale purification protocol for butyrylcholinesterase based on ammonium sulfate fractionation followed by an batch affinity chromatography should be applicable also for acetylcholinesterase with minor modifications. See, for example, Grunwald *et al., J. Biochem. Biophys. Methods* 34: 123-135 (1997), the complete disclosure of which is hereby incorporated herein by reference.

Additional purification schemes, which are well known in the art, involve engineering a tag to the recombinant enzyme by creating translational fusions. Commercially available plasmids directing such fusions exist mainly for bacterial expression, but can easily be adapted to expression in plants. For example, well known tags that can be used include histidine tags (whereby purification is typically conducted by a nickel-based affinity chromatography); intein-chitin binding tags (whereby purification is conducted by chitin based affinity chromatography and cleavage by a reducing agent, such as dithiothreitol or beta-mercaptoethanol); cellulose binding domains (whereby purification involves affinity chromatography with cellulose). The latter is likely the most useful approach, as it can be done without the addition of any exogenous affinity matrix,

since the cholinesterase-CBD fusion binds to cell walls of the plant extract. Release is then mediated by either addition of cellobiose or brief acidification. Cleavage is also possible. For some applications, the cellulose immobilized enzyme-CBD will be extremely useful as a catalytic platform for filters, cellulose based cleaning aids *etc.*

Further examples of applications of the invention

Administration of exogenous cholinesterases is an efficacious and safe treatment for the prevention of anti-AChE toxicity. In fact, a single pre-treatment injection of either AChE or BuChE may be sufficient for full protection without any post-exposure treatment. However, to ensure maximum protection against a high dosage (equal to several LD50) of OPs, large amounts of the enzymes are required to satisfy the 1:1 stoichiometry required between the enzyme and the inhibitors in the blood. The enzymes can be purified from human or animal blood, or alternatively, they can be expressed in a variety of cell cultures. However, these systems inherently suffer from high costs and risks of contamination with human pathogens. Recombinant cholinesterases of various sources have been expressed in *Escherichia coli*, however, the enzymes thus produced must be denatured and refolded to obtain even partial activity. In addition, they are very labile as compared to the native enzymes. Production by fermentation of yeast cell cultures is also possible, but costs are high and scaling up is expensive.

Therefore, we introduced transgenic plants as a novel production system for human acetylcholinesterase, a key component of cholinergic synapses. Some of the transgenic tomato lines obtained express high levels of AChE activity, with accumulation levels (on a fresh weight basis) as reported for the yeast-derived enzyme (Figures 1 and 2). This activity represents authentic human acetylcholinesterase activity, as judged by its enzymatic properties (Figures 3 and 4). The plant-derived enzyme is also very stable in the crude plant extract (Figures 5A and 5B). Expression levels of the gene product can be increased further by optimizing the coding sequence of the human gene for expression in plants, according to methods that are well known in the art, and by regulating and restricting expression of the gene product to certain tissues.

In humans, AChE is encoded by a single gene which yields, through alternative splicing of its pre-mRNA, three polypeptide isoforms with distinct C-termini. We

expressed the engineered AChE-E4 form, encoded by exons 2-4 of the human gene. However, one of ordinary skill in the art will appreciate that the other isoforms can be used as well. AChE-E4 consists of the globular N-terminal domain shared among the three physiological variants of AChE. Expressed by itself, the soluble AChE-E4 polypeptide is a fully competent acetylcholine hydrolase with kinetic properties which are similar to those of the natural forms. This recombinant AChE-E4 variant is especially suited for application as a protective decoy for the neutralization of AChE inhibitors, because its kinetic properties are practically identical to those of synaptic AChE (unlike BuChE). Because it is soluble, it may be cleared more slowly from circulation (unlike the membrane bound AChE forms, which are cleared 50 times faster than soluble BuChE). Because it has the same amino acid sequence as the human enzyme, the plant-derived recombinant hAChE-E4 isoform is expected to be less immunogenic than the heterologous cholinesterases used in previous studies. There are three potential glycosylation sites in human AChE, and glycosylation, which does not affect the enzymatic properties of the enzyme, is important for both the stability of AChE and its pharmacokinetics and its immunogenic properties. As eukaryotes, plants offer the advantage of all forms of post-translational modification, including glycosylation, which, however, differs in details from that in mammals.

Use of the inexpensively produced enzyme is not limited to application by injection, as efficacy of other routes of entry into the body (*e.g.*, orally, inhalation) is expected as well. Lastly, cholinesterases can be incorporated into cleansing preparations, protective skin-creams, filtration devices, and biosensors. For these purposes, the plant-derived enzyme is especially useful, due to lower costs of partial purification and its higher stability.

The extensive use of anticholinesterase pesticides and the concurrent accidental poisoning, the unfortunate threat of OP chemical warfare agents by terrorists and rogue governments, as well as environmental concerns, are the driving force for the development of effective, inexpensive and safe countermeasures and bioremediation solutions. Plant-derived recombinant human AChE is an important step in this direction.

Accordingly, it is to be understood that the embodiments of the invention herein described are merely illustrative of the application of the principles of the invention. Reference herein to details of the illustrated embodiments is not intended to limit the scope of the claims, which themselves recite those features regarded as essential to the invention.